Marshall Lab DamID-seq protocol (version 2016-Sep-29)

This protocol is based on the following published research:
Marshall OJ, Southall TD, Cheetham SW, Brand AH (2016) *Nat Protocols* 11:1586–1598
Marshall OJ, Brand AH (2015) *Bioinformatics* 31:3371-3
Southall TD, Gold KS, Egger B, Davidson CM, Caygill EE, Marshall OJ, Brand AH (2013) *Dev Cell* 26:101-12
Vogel MJ, Peric-Hupkes D, van Steensel B (2007) *Nat Protoc* 2:1467–1478.

Buffer AL protocol (Qiagen DNA Micro kit)
(drilling protocol: recommended for whole embryos, whole adult heads)

1. Take Dam embryos or dissected tissue from -80°C freezer (stored without buffer)
2. Add 140 µL 1X PBS + 40µL 500mM EDTA into eppendorf tube to wash and mix the embryos (if insufficient embryos in one tube, you can take embryos from several tubes and put together into one tube).
   NB: Addition of EDTA is optional, but recommended when working with tissue containing the gut (or any tissue with high concentrations of nucleases/proteases). We have not tried lower concentrations than 100mM; lower concentrations may also work.
3. Add 20 µL RNase (12.5µg/µL) and pipette mix
4. Use sterile (wash in 100% ethanol) pestle to homogenise samples (using electric drill)
5. Add 20 µL Proteinase K (Qiagen DNeasy Kit); gently pipette up and down and leave for 1 min at RT
6. Add 200µL Buffer AL (Qiagen DNeasy Kit); gently pipette up and down with blue tip - mix ~50 times and put at 56°C for 10 mins (heat block)
7. Cool to RT, add 200 µL 100% ethanol and mix (gently pipette with blue tip)
8. Continue immediately to DNA extraction

Alternative Buffer ATL protocol (Qiagen DNA Micro kit)
(no drilling: recommended for torn larvae or dissected tissue)

1. Take cut larvae or dissected tissue from -80°C freezer (stored without buffer)
2. Add 20µL 500mM EDTA (50mM final conc)
   NB: Addition of EDTA is optional, but recommended when working with tissue containing the gut (or any tissue with high concentrations of nucleases/proteases). We have not tried lower concentrations than 50mM; lower concentrations may also work.
3. Add 180µL ATL buffer
4. Add 20µL Proteinase K (Qiagen DNeasy Kit), mix by flicking gently
5. Incubate at 56°C on heat block until digested; flick gently to mix occasionally
   Digestion can take from one hour (dissected brains) to overnight (torn larvae). Qiagen claims that samples can be stored at RT for up to 6 months following Proteinase-K digestion in this buffer without degradation.
6. Cool to RT and add 20 µL RNase (12.5ug/µL)
7. Incubate at RT for 2 mins
8. Mix 200µL Buffer AL and 200µL 100% EtOH together (per sample) in separate tube; mix well by vortexing
9. Add 400µL of Buffer AL/EtOH mix to each sample; mix well by inversion and gentle flicking

DNA extraction (Qiagen DNA Micro kit)

Don't worry about the spin speeds here – maximum speed in a benchtop centrifuge is fine!
(To increase throughput, a Qiavac vacuum manifold can be used instead of centrifuging. However, if using a vacuum manifold, be sure to still perform the final 3 min spin step to dry to columns.)

1. Add all solution to a spin column
2. Spin at >6000 x *g* for 1 min, discard flow-through and collecting tube.
3. Add 500µl AW1 solution and spin at >6000 x *g* for 1 min, discard flow-through and collecting tube
4. Add 500µl AW2 solution and spin at >6000 x *g* for 1 min, discard flow-through and collecting tube
5. Transfer column to a new tube and spin at 20,000 x *g* for 3 mins to dry the column
6. Transfer spin column to a new 1.5 ml tube, add 50 µl AE buffer and leave at RT for at least 30 min. Spin at >6000 x *g* for 1 min and keep the flow-through
7. Run 1µl on a 0.8% gel to check quality (should be a single band on the top of gel and not a smear)

DpnI digestion

1. Transfer 43.5µl of elution to a new 1.5ml tube
2. Add 5µL NEB buffer 4 / SmartCut and 1.5µl DpnI enzyme, mix very gently with blue tip and digest at 37°C overnight.
   (This digestion step can be reduced to two hours if required.)
   DpnI can be optionally heat-inactivated for 20 mins at 80°C, but this is unnecessary as the enzyme will be removed by the PCR clean-up below.

3. Clean-up with Qiagen PCR Purification kit (250µL Buffer PB); elute in 32µL DEPC H_2O [Label tube "A"]
4. Measure DNA conc on Qubit or Nanodrop and dilute samples to a maximum concentration of 750ng (if lower, use undiluted) in 15µL DEPC H_2O in a PCR strip (store the remainder at -20°C for future use).
   Less DNA yield at this point is often better – note that uncut genomic DNA (which should the the majority of DNA in most use cases) should not pass through the spin column, so the purified DNA should only come from induced cells. NB: very low or even undetectable yields at this stage can produce excellent data – do not throw samples away at this point!

Adaptor ligation

1. Add 4µL Adaptor Ligation Buffer (AL)
2. Add 1µL T4 DNA ligase and mix well
3. Ligate for 2 hours (16°C for 2 hours → 65°C for 10 mins in PCR machine)

DpnII digestion

1. Add 19µL DpnII Digestion Buffer (DIIB)
2. Add 1µL DpnII enzyme and mix well
3. Digest at 37°C for 2 hours
4. Heat inactivate at 65°C for 20 mins

PCR amplification

1. Add 118µL PCR Buffer (PCR)
2. Add 2µL MyTaq polymerase and mix well
3. Split into 4 x 40µL reactions in a PCR strip using multichannel
4. Run PCR (use DamID 17 programme in PCR machine):

```
68°C 10 min
94°C 30 sec
65°C 5 min
68°C 15 min
Repeat 3x
      94°C 30 sec
      65°C 1 min
      68°C 10 min
Repeat 17x
      94°C 30 sec
      65°C 1 min
      68°C 2 min
68°C 5 min
4°C hold
```

This PCR protocol is from Vogel et al.*, 2007. Both the initial long extension for 10 mins and the subsequent long cycles appear to be required. A 3 min extension time, as present in the final 17 cycles, should amplify all fragments below 5kb; these represent 99.97% of all GATC fragments in the* D. melanogaster *genome.*

(An argument could be made that fragments larger than 5kb will not represent genuine binding at that fragment, given Dam-fusion proteins typically only methylate within +/- 2kb of their binding site in a seemingly normal distribution. On that basis, longer amplification times should probably be discouraged.)

QC and sonication

1. PCR purify (160µL sample + 800µL Buffer PB). If using MyTaq, wash column twice with PE Buffer before elution. Use 32 µL DEPC H_2O to elute (leave water on column for at least 15 min) [Label tubes "C"]
2. Run 1µL on a gel to check quality (should be a smear between ~400bp and 2kb)
3. Spec DNA conc on nanodrop
4. Dilute samples: 2µg DNA in 90µL DEPC H_2O in 1.5mL TPX sonication tube
   Using dedicated sonication tubes appears to be important in order to obtain consistent results
5. Add 10µL CutSmart Buffer and mix well; cool on ice
6. Sonicate in Diagenode Bioruptor Plus (in cold room):
   a. Switch on (both cooling and head units) at least 10 mins before use – wait until water temp is 4°C
   b. Sonicate for 5-6 cycles (30 secs on, 30 secs off) on high power
      The aim is to achieve a ~300bp average fragment size. All sonicators are different; if using a different sonication device you will need to optimise the conditions first.
7. Check fragment size on Tapestation (genomic tape) – ensure that average fragment size is ~300bp
   Slight variations in fragment size are acceptable. Very large fragments (>600bp) may impede clustering efficiency and sequencing yields
8. Add 1µL AlwI enzyme and digest overnight at 37°C
   AlwI removes the DamID adaptors and initial GATC sequence (cuts at GGATCNNNN\|N). Digestion can be carried out either before or after sonication; it makes no difference to the end result. AlwI cannot be heat inactivated, but will not cause issues if carried over in subsequent steps.
9. Transfer 70µL (or 90µL) of each sample into PCR 8-well strips for library prep

Library preparation

The following protocol uses homebrew reagents and is not compatible with Illumina kits. If you wish to use a commercial Illumina kit, we recommend the TruSeq Nano kit – follow the manufacturer's protocol except for the PCR step, in which case still use a reduced number of cycles (6 cycles seems optimum for most cases – see notes below). Commercial AmpureXP beads are a (vastly more expensive) alternative to homemade Seramag beads.

Purify with Seramag beads

1. Add 105µL of Seramag beads to 70µL sample (or 135µL to 90µL) and mix well
   Pipette mix 20x and then flick mix the strip and pulse down. Rapid mixing of beads and sample is important. Use 90µL of samples if concentration prior to sonication was low.
2. Incubate at RT, 10'
3. Place on magnetic stand 10' (or until clear)
4. Remove supernatant
5. Wash twice in 190µL 80% EtOH (30 secs wash time)
6. Let plate stand for 5' to air dry
7. Resuspend in 25µL Resuspension Buffer and remove from magnetic stand
8. Mix well and incubate 2' RT
9. Place on magnetic stand for 5' (or until clear)
10. Put 22.5µL of the supernatant into a new, clean tube for the next step

Adjust concentrations

1. Measure library conc on Qubit
2. Dilute samples to (no more than) 500ng of DNA in 20µL Resuspension Buffer

End repair

1. Add 7.5µL End Repair Buffer (ERB)
2. Add 2.5µL End Repair enzyme and mix well
3. Run PCR program "Truseq 1 2 ER Hi":
    a. Incubate for 30 mins at 30°C
    b. Heat inactivate enzymes for 20 mins at 75°C

Adenylate 3' Ends

1. Add 0.75µL Klenow 3' to 5' exo- enzyme and mix well
2. Run PCR program "Truseq 2 AT":
    a. Incubate for 30 mins at 37°C
3. Proceed immediately to adaptor ligation

Adaptor ligation

1. Add 2.5µL NEB Quick Ligase enzyme
2. Add 2.5µL of relevant adaptor
3. Run PCR program "Truseq 3 lig":
    a. Incubate for 10 mins at 30°C
4. Add 5µL Stop Ligation Buffer

Clean-up with Seramag beads

1. Add 40µL Seramag beads and mix well
2. Incubate at RT, 10'
3. Place on magnetic stand 5' (or until clear)
4. Remove supernatant
5. Wash twice in 190µL 80% EtOH (30 secs wash time)
6. Let plate stand for 5' to air dry
7. Resuspend in 52.5µL Resuspension Buffer and remove from magnetic stand
8. Mix well and incubate 2' RT
9. Place on magnetic stand for 5' (or until clear)
10. Put 50µL of the supernatant into a new, clean tube for the next step

Clean-up with Seramag beads again

1. Add 50µL Seramag beads and mix well
2. incubate at RT, 10'
3. Place on magnetic stand 5' (or until clear)
4. Remove supernatant
5. Wash twice in 190µL 80% EtOH (30 secs wash time)
6. Let plate stand for 5' to air dry
7. Resuspend in 22.5µL Resuspension Buffer and remove from magnetic stand
8. Mix well and incubate 2' RT
9. Place on magnetic stand for 5' (or until clear)
10. Put 20µL of the supernatant into a new, clean tube for the next step
    This second clean-up is required to ensure complete removal of sequencing adaptor dimers – should these be present in the final library they will vastly out-compete genuine library sequences when hybridising to the flow cell.

Enrich DNA fragments

1. Add 5µL PCR Primer Cocktail
2. Add 25µL PCR Master Mix
3. Run PCR program "Truseq 4 PCR 6 cycles":

```
98°C for 30 secs
6 cycles of:
      98°C for 10 secs
      60°C for 30 secs
      72°C for 30 secs
72°C for 5 mins
```

   Note: aim to avoid concatamer formation (see troubleshooting section below)

Clean-up with Seramag beads (final time)

1. Add 50µL Seramag beads and mix well
2. incubate at RT, 10'
3. Place on magnetic stand 5' (or until clear)
4. Remove supernatant
5. Wash twice in 190µL 80% EtOH (30 secs wash time)
6. Let plate stand for 15' to air dry
7. Resuspend in 32.5µL Resuspension Buffer and remove from magnetic stand
8. Mix well and incubate 2' RT
9. Place on magnetic stand for 5' (or until clear)
10. Put 30µL of the supernatant into a new clean tube

Library QC and multiplexing

1. QC sample with tapestation (use genomic tape) – ensure that concatemers are not present (see "Troubleshooting" below) and that adapter dimers are not present (small peak around 240bp) in the sample. Record the average fragment length for each sample.
2. Measure DNA conc with Qubit (see attached protocol)
3. Calculate molarity of each sample from fragment length and concentration – use the spreadsheet template (ask Owen for this), or see the "Using the Qbit" section for details as to how to calculate molarity manually
4. Pool samples to give 20nM final DNA conc (with all libraries at equal concentration) – use the spreadsheet template to calculate this automatically
5. Sequence as single-end 50nt (SE50) on Illumina sequencer.

Bioinformatic processing of samples

1. Log_2(Dam-fusion/Dam) binding profiles can be obtained from either raw reads in FASTQ format or aligned BAM files using the damidseq_pipeline software, freely available from https://owenjm.github.io/damidseq_pipeline. Detailed usage instructions and examples are available from the website.
2. Profiles can be viewed using browser software such as IGV, as detailed on the website.
3. Calling expressed genes from RNA pol II ratio files generated by the damidseq_pipeline software can be carried out using the polii.gene.call.r script, freely available from https://github.com/owenjm/polii.gene.call.

Notes

1. Wear gloves, use filter tips and clean, autoclaved H_2O to avoid contamination
   Exceptions to filter tips: DNeasy/Qiagen kits before final elution
   Always use filter tips when preparing sequencing libraries
   The use of DEPC H_2O throughout this protocol, while technically unnecessary, reflects the need to use a clean, contamination-free source of H_2O

2. **Be very gentle with samples prior to DpnI digestion.** Any genomic DNA shearing will result in broken ends that can potentially ligate DamID adapters. This will lead to random signals. Do not vortex, but mix samples gently by inversion and/or slow pipetting with a blue tip (and/or *very* gentle flicking). Always check the quality of the DNeasy-prepped DNA on a gel before continuing with the protocol.

3. For forward planning, each sample uses 278.5µL beads during the sequencing library prep

Citations

If you find this protocol useful, please cite
http://www.ncbi.nlm.nih.gov/pubmed/23792147 (original TaDa publication) and
http://www.ncbi.nlm.nih.gov/pubmed/26112292 (bioinformatic processing and NGS method); or
http://www.ncbi.nlm.nih.gov/pubmed/27490632 (Nature Protocols)

Ligation of DamID adaptors

Making ds AdR stock (50uM):

1. Take 50 µL AdRt (100uM) and 50 µL AdRb (100 uM)
2. Incubate in removable metal heating block, 95°C, 2 mins
3. Remove heating block and allow to cool to room temperature (should take > 45mins)

DamID adaptor sequences

AdRt

```
CTAATACGACTCACTATAGGGCAGCGTGGTCGCGGCCGAGGA
```

AdRb

```
TCCTCGGCCG
```

Annealed structure:

```
5' CTAATACGACTCACTATAGGGCAGCGTGGTCGCGGCCGAGGA 3'
                                   ||||||||||
                                3' GCCGGCTCCT 5'
```

Primer sequences

DamID_PCR

```
GGTCGCGGCCGAGGATC
```

Reagents

MyTaq HS DNA polymerase

Bioline, Cat# BIO-21112 (200µl) or BIO-21113 (500µl)

Note: it's been hard to find a replacement for the old Clontech Advantage cDNA polymerase. MyTaq HS seems the best so far; Clontech's Advantage 2 will work, but at reduced yield and is not recommended. Another, untested, alternative would be Cesium Klentaq AC LA from DNA Polymerase Technology.

Seramag SpeedBead, 3 EDAC/PA5, 1µm, 15mL

Fisher Scientific, Cat# 12326433

Note: Seramag beads need to be diluted in PEG before use as described in the dedicated section below. AmpureXP beads (which are ready to use and do not need further preparation) may be substituted for Seramag beads.

NEBNext® High-Fidelity 2X PCR Master Mix 1.25mL (NEB, Cat# M0541S)

Alternative to NEBNext PCR MM (works equally well, but may be more expensive):

Kapa HiFi HS ReadyMix 1.25mL (Anachem, Cat# KK2601)

Quick Ligase (NEB, Cat# M2200S)
T4 ligase (NEB, Cat# M0202S)
T4 DNA polymerase (NEB, Cat# M0203S)
T4 polynucleotide kinase (NEB, Cat# M0201S)
Klenow fragment (NEB, Cat# M0210S)
Klenow 3' to 5' exo- (NEB, Cat# M0212L)
NEB restriction enzymes: DpnI (Cat# R0176L), DpnII (Cat# R0543L), AlwI (Cat# R0513L)
dNTPs (NEB, Cat# N0447S)

DamID buffers

DpnI digestion buffer (DI)		**x100**	**Aliquot**
Buffer 4	5	500	505
H2O	43.5	4350	

Ligation buffer (AL)		**x100**	**Aliquot**
10x Ligation buffer	2	200	40
0.8uL ds ADR	0.8	80	
H2O	1.2	120	

DpnII digestion buffer (DIIB)		**x100**	**Aliquot**
Dpn II Buffer	4	400	190
H2O	15	1500	

PCR (MyTaq HS)		**x50**	**Aliquot**
5X MyTaq HS Buffer	32	1600	1180
DamID-PCR primer (50 µM)	2.5	125	
DEPC H2O	83.5	4175	

(Alternative PCR, Advantage 2)		**x50**	**Aliquot**
10X PCR buffer (Clontech)	16	800	1180
DamID-PCR primer (50 µM)	2.5	125	
50X dNTP mix	3.2	160	
DEPC H2O	96.3	4815	

(note: 50x dNTP mix = 10mM each of dATP, dTTP, dCTP, dGTP; final conc. is 0.2mM each)

10x T4 ligase buffer (supplied with enzyme)

500 mM Tris-HCl
100 mM $MgCl_2$
10 mM ATP
100 mM DTT
pH 7.5 @ 25°C

Homebrew sequencing buffers

The following buffers and components are mostly adapted from Ethan Ford's protocol

End Repair Buffer	**1x (7.5µL)**	**Master mix x50**
NEB T4 ligase buffer 10x	3.0	150
10mM dNTPs	1.2	60
DEPC H_2O	3.3	165
Total	**7.5**	**375**

End Repair enzymes	**1x (2.5µL)**	**Master mix x50**
T4 DNA polymerase (NEB, 3U/µL)	1.14	56.82
Klenow Fragment (NEB, 5 U/µL)	0.23	11.36
T4 polynucleotide kinase (NEB, 10 U/µL)	1.14	56.82
Total	**2.5**	**125.00**

PCR Master Mix

NEBNext 2x HiFi Hotstart Ready-mix (aliquot into 105µL aliquots and use as supplied)

PCR Primer Cocktail

25 µL PCR1 primer (100 µM)
25 µL PCR2 primer (100 µM)
50 µL DEPC H_2O

Resuspension Buffer

10mM Tris-HCl, pH 8.0
0.1 mM EDTA

Ligation Stop Solution

0.5M EDTA

Sequencing adaptors and primer sequences

(adaptor barcodes highlighted in red)

Universal	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATC*T
Index 1	[Phos]GATCGGAAGAGCACACGTCTGAACTCCAGTCAC**ATCACG**ATCTCGTATGCCGTCTTCTGCTT*G
Index 2	[Phos]GATCGGAAGAGCACACGTCTGAACTCCAGTCAC**CGATGT**ATCTCGTATGCCGTCTTCTGCTT*G
Index 3	[Phos]GATCGGAAGAGCACACGTCTGAACTCCAGTCAC**TTAGGC**ATCTCGTATGCCGTCTTCTGCTT*G
Index 4	[Phos]GATCGGAAGAGCACACGTCTGAACTCCAGTCAC**TGACCA**ATCTCGTATGCCGTCTTCTGCTT*G
Index 5	[Phos]GATCGGAAGAGCACACGTCTGAACTCCAGTCAC**ACAGTG**ATCTCGTATGCCGTCTTCTGCTT*G
Index 6	[Phos]GATCGGAAGAGCACACGTCTGAACTCCAGTCAC**GCCAAT**ATCTCGTATGCCGTCTTCTGCTT*G
Index 7	[Phos]GATCGGAAGAGCACACGTCTGAACTCCAGTCAC**CAGATC**ATCTCGTATGCCGTCTTCTGCTT*G
Index 8	[Phos]GATCGGAAGAGCACACGTCTGAACTCCAGTCAC**ACTTGA**ATCTCGTATGCCGTCTTCTGCTT*G
Index 9	[Phos]GATCGGAAGAGCACACGTCTGAACTCCAGTCAC**GATCAG**ATCTCGTATGCCGTCTTCTGCTT*G
Index 10	[Phos]GATCGGAAGAGCACACGTCTGAACTCCAGTCAC**TAGCTT**ATCTCGTATGCCGTCTTCTGCTT*G
Index 11	[Phos]GATCGGAAGAGCACACGTCTGAACTCCAGTCAC**GGCTAC**ATCTCGTATGCCGTCTTCTGCTT*G
Index 12	[Phos]GATCGGAAGAGCACACGTCTGAACTCCAGTCAC**CTTGTA**ATCTCGTATGCCGTCTTCTGCTT*G
Index 13	[Phos]GATCGGAAGAGCACACGTCTGAACTCCAGTCAC**AGTCAA**ATCTCGTATGCCGTCTTCTGCTT*G
Index 14	[Phos]GATCGGAAGAGCACACGTCTGAACTCCAGTCAC**AGTTCC**ATCTCGTATGCCGTCTTCTGCTT*G
Index 15	[Phos]GATCGGAAGAGCACACGTCTGAACTCCAGTCAC**ATGTCA**ATCTCGTATGCCGTCTTCTGCTT*G
Index 16	[Phos]GATCGGAAGAGCACACGTCTGAACTCCAGTCAC**CCGTCC**ATCTCGTATGCCGTCTTCTGCTT*G
Index 18	[Phos]GATCGGAAGAGCACACGTCTGAACTCCAGTCAC**GTCCGC**ATCTCGTATGCCGTCTTCTGCTT*G
Index 19	[Phos]GATCGGAAGAGCACACGTCTGAACTCCAGTCAC**GTGAAA**ATCTCGTATGCCGTCTTCTGCTT*G
PCR1	AATGATACGGCGACCACCGA*G
PCR2	CAAGCAGAAGACGGCATACGA*G

[Phos] = 5′ Phosphorylation
* = Phosphorothioate linkages

Annealing adaptors

1. Resuspend adaptor oligos at 100µM in TE + 50mM NaCl
2. Mix 25µL of relevant index + 25µL Universal primer in PCR tube
3. Incubate in removable metal heating block, 95°C, 2 mins
4. Remove heating block and allow to cool to room temperature (should take > 45mins)

Notes re. adaptors

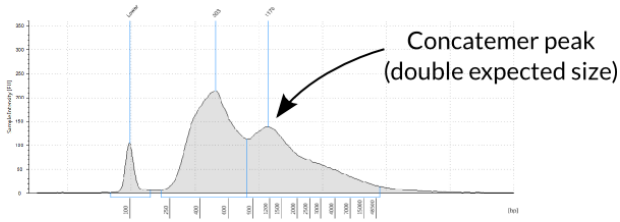
1. If multiplexing four or fewer libraries, selecting adaptors with barcodes that are too similar may result in a reduced number of reads passing the filter. In this case, the preferred indexes to use (in order) are 4, 7, 6 and 8.

Troubleshooting

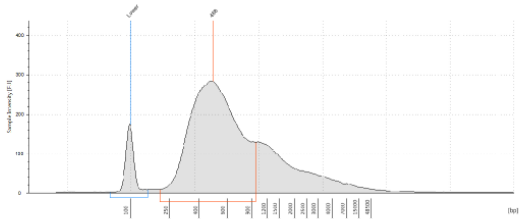
Secondary peak after PCR

This generally only happens using 8 or 10 (or more) cycles as PCR reaction is exhausted – a secondary peak twice the size of the original peak is seen on the tapestation genomic plots. This peak results from concatemers of the amplified product. If you see this, reduce number of PCR cycles and try again (6 cycles seems to work well for TaDa).

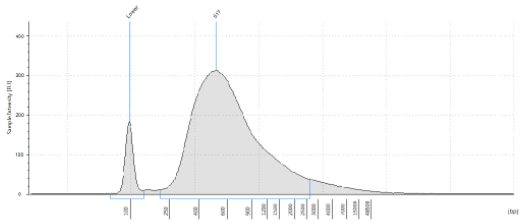
10 cycles



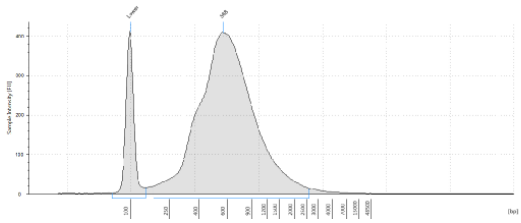
8 cycles



6 cycles



Input to PCR



Preparation of Seramag beads

(*modified from* Rohland N and Reich D. (2012) Cost-effective, high-throughput DNA sequencing libraries for multiplexed target capture. *Genome Res* **22**:939–46. doi:10.1101/gr.128124.111)

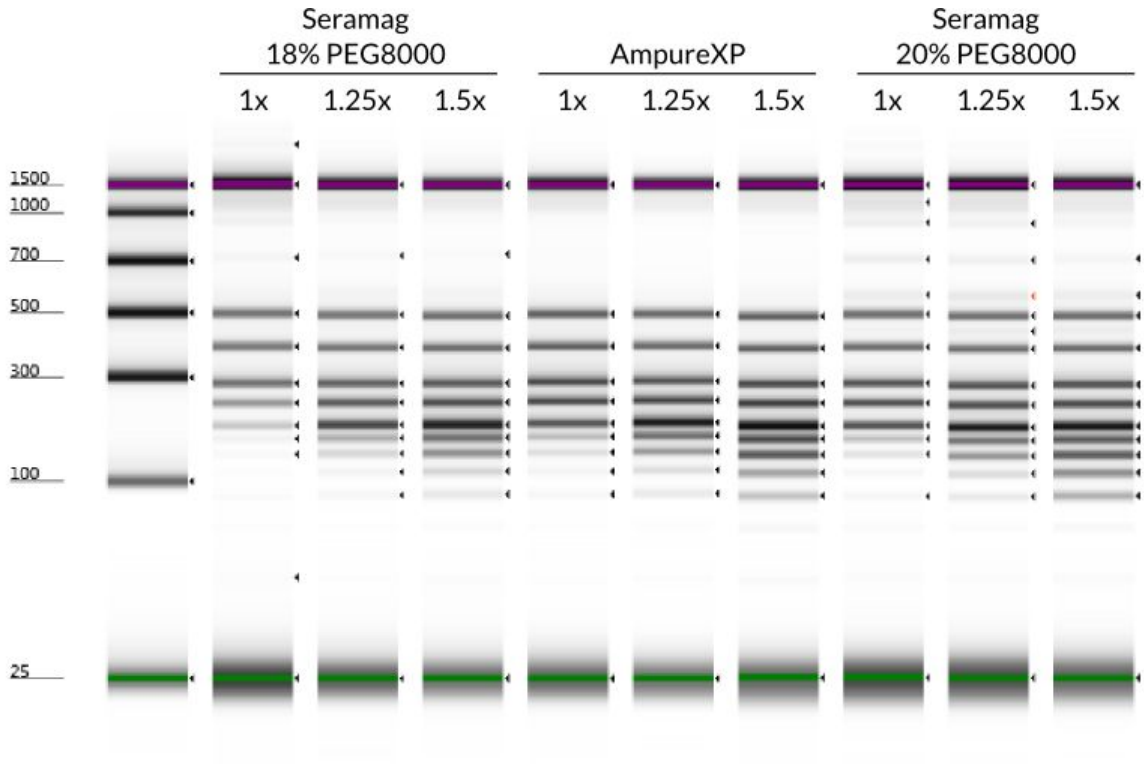
1. In 15mL Falcon tube add:

   3g PEG-8000 (20% w/v final conc)
   3mL 5M NaCl (1M final)
   150µL 1M Tris pH 8.0 (10mM final)
   30µL 0.5M EDTA (1mM final)

2. add DEPC H_2O to 14mL
3. mix by inversion until PEG is disolved (~5mins)
4. mix Speedbeads container well to ensure beads are in suspension
5. add 300µL Seramag Speedbeads and mix by inversion
6. add DEPC H20 to 15mL volume and store at 4°C

<u>Test beads by purifying Bioline Hyperladder V</u>

1. Make mastermix of diluted ladder: 10µL ladder + 20µL DEPC H_2O per sample
2. Purify ladder with 1x, 1.25x, 1.5x amounts of beads
3. Elute in 20µL DEPC H_20
4. Run on tapestation using D1K tape (should look like 20% PEG-8000 lanes):



Notes

1. Rohland and Reich used 18% PEG-8000; however, this does not appear to perform as well as 20% in ladder purification (compared with AmpureXP)

2. Based on current prices:
   Ampure beads are £689.67 for 60mL working solution
   Seramag beads are £317.62 for 15mL, which makes 750mL working solution
   Seramag beads are therefore more than 27x more cost-effective

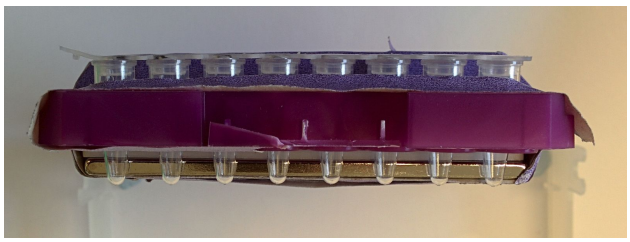
Optional: homemade magnetic rack for bead purification

Thermoscientific provide a 96-well magnetic plate for £763, which works reasonably well. However, the total retail cost of the neodymium magnets in this plate is ~£6 (!)

Whilst it would be possible to buy individual ring magnets and glue them to a 96-well rack with epoxy resin, I've found it easier to make a simple 8-well (i.e. one PCR strip) magnetic plate using:

1x N42 Neodymium magnet (75x10x3mm) (e.g. these magnets)
1x 20µL filter tip box insert (acting as the 96-well support)
1x 75x10x2.5mm plastic spacer (supplied with a pair of the strip magnets)

The magnet together with spacer is taped to the tip box insert as shown below.



Important: as the magnet needs to remain firmly in contact with the edge of the strip tubes at all times, use a second PCR strip and/or tape on the side of the strip to hold the magnet in place.

The advantage of using a strip magnet rather than a circular magnet is that the beads are pulled to one side of the tube rather than sitting in a ring, greatly facilitating liquid removal. (The disadvantage is that such a configuration is not compatible with 96-well high-throughput solutions).

Strength N42 seems to work well, but there should be no harm in using more powerful magnets if you can obtain them (the only result will be a faster purification process).

Using the tapestation

1. Prepare ladder and samples in 8-well PCR strips:
   2µL ladder in lane 1
   3µL sample buffer + 1µL sample in subsequent lanes
   (the Tapestation suggests 3µL for the ladder, but smaller volumes are fine and save a substantial amount of money. The ladder can also be diluted 1:20 rather than 1:10, although this will obviously affect quantitation if this is required.)
2. Cap strip and mix well by flicking (pulse down and mix several times)
3. Turn on tapestation and laptop
4. Launch Tapestation software and wait until prompted to insert the tape
5. Open tapestation, place tape in slot in correct orientation (barcode towards you on the right)
   We recommend using genomic tapes rather than D1K HS tapes – the genomic tapes give a better picture of adaptor-concatemer formation if present. D5K tapes may present an even better solution here, but we haven't tried these yet.
6. Insert strip with ladder and samples and loading tips and close the machine
7. Select the wells you want to run on the software, and add labels in the table that appears
8. Click "Start" to begin the run (will take ~10-20 min to complete)
9. Once run is complete and analysis software is open, record average fragment sizes and save a report

Using the Qbit

1. Prepare samples as per the included protocol.
2. NB – use 1µL sample + 199µL working solution for each sample
3. With each sample, press the button to obtain the DNA concentration
4. DNA molarity $\cong \frac{1500}{(\textit{fragment size in bp})} * [\textit{conc in ng}/\mu L]$
5. Use a spreadsheet template to calculate final pooling of samples

Qubit™ Assays

QUICK REFERENCE CARD

www.invitrogen.com/qubit

NOTE: For best results, store the dye and the buffer at room temperature. Store the DNA, RNA, and protein standards at 4°C. Ensure that all assay reagents are at **room temperature** before you begin.

1. Set up two Assay Tubes for the standards (three for the protein assay) and one tube for each user sample.
2. Prepare the Qubit™ **Working Solution** by diluting the Qubit™ reagent 1:200 in Qubit™ buffer. Prepare 200 µL of **Working Solution** for each standard and sample.
3. Prepare the Assay Tubes* according to the table below.

	Standard Assay Tubes	**User Sample** Assay Tubes
Volume of **Working Solution** (from step 2) to add	190 µL	180–199 µL
Volume of **Standard** (from kit) to add	10 µL	—
Volume of **User Sample** to add	—	1–20 µL
Total Volume in each Assay Tube	200 µL	200 µL

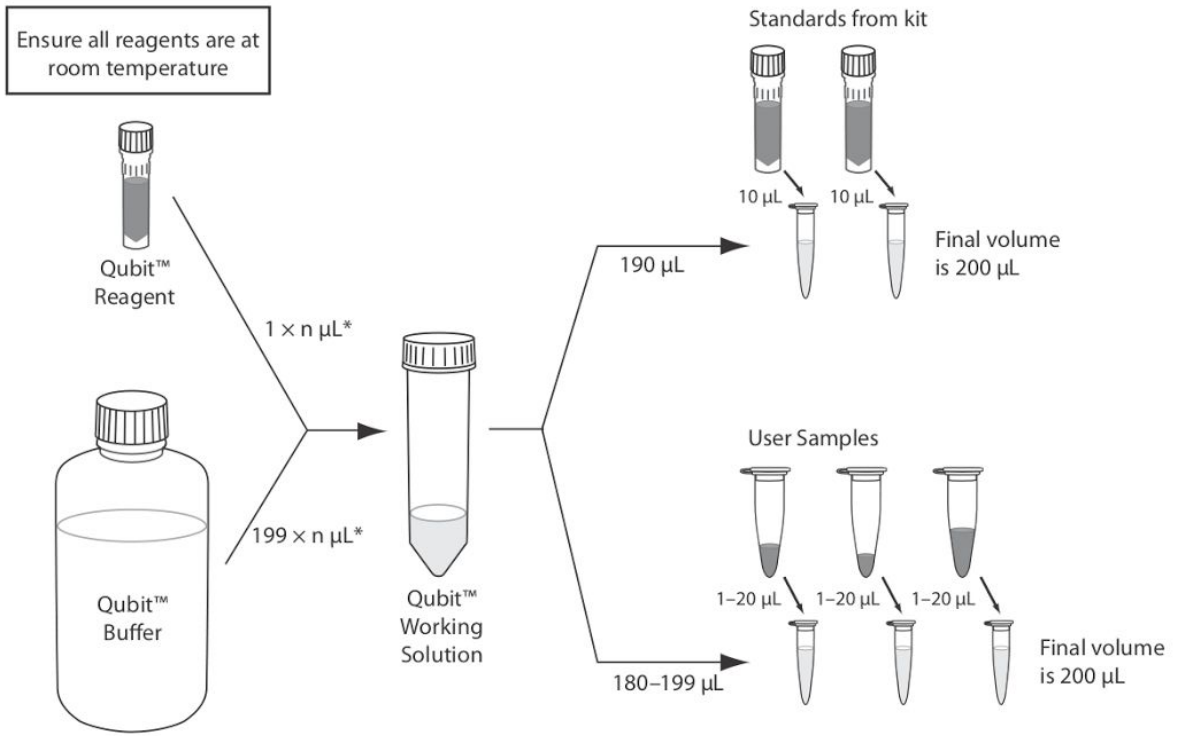
* Use only thin-wall, clear 0.5 mL PCR tubes. Acceptable tubes include Qubit® assay tubes (set of 500, Cat. no. Q32856) or Axygen PCR-05-C tubes (VWR, part no. 10011-830).

4. Vortex all tubes for 2–3 seconds.
5. Incubate the tubes for 2 minutes at room temperature (15 minutes for the Qubit™ protein assay).
6. Insert the tubes in the Qubit® 2.0 Fluorometer and take readings. For detailed instructions, refer to the Qubit® 2.0 Fluorometer manual.
7. *Optional:* Using the Dilution Calculator feature of the Qubit® 2.0 Fluorometer, determine the stock concentration of your original sample.

MP39808 | MAN0002474
Rev. date: 28 Sept 2010

molecular probes® | invitrogen™
by life technologies™

For technical support, email tech_support@invitrogen.com.
For country-specific contact information, visit www.invitrogen.com.



* where n = number of Standards plus number of Samples

molecular probes | invitrogen
by life technologies

Vortex all assay tubes for 2–3 seconds

Incubate at room temperature for 2 minutes (15 minutes for Qubit™ protein assay)

Read tubes in Qubit® 2.0 Fluorometer